RAGE Does Not Affect Amyloid Pathology in Transgenic ArcAbeta Mice

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Abstract: Background: Alzheimer's disease (AD) is characterized by brain accumulation of the amyloid-beta peptide (Abeta) that triggers a cascade of biochemical and cellular alterations resulting in the clinical phenotype of the disease. While numerous experiments addressed Abeta toxicity, the mechanisms are still not fully understood. The receptor for advanced glycation end products (RAGE) binds Abeta and was suggested to be involved in the pathological processes of AD. Objective: Our purpose was to assess the effect of RAGE deletion on Abeta-related pathology. Methods: We crossed RAGE knockout (RAGE(-/-)) mice with transgenic mice harboring both the Swedish and Arctic Abeta precursor protein mutations (arcAbeta mice). We assessed Abeta levels, Abeta brain deposition, Abeta-degrading enzyme activities, Abeta precursor protein expression and processing, number and morphology of microglia as well as cognitive performance of 6- and 12-month-old RAGE(-/-)/arcAbeta, RAGE(-/-), arcAbeta and wild-type mice. Results: RAGE(-/-)/arcAbeta mice had significantly lower levels of SDS- and formic-acid-extracted Abeta in the cortex and hippocampus, with concomitantly increased activity of insulin-degrading enzyme at the age of 6 months. However, RAGE deletion could neither prevent the decline in cognitive performance nor the age-related cerebral accumulation of Abeta peptide. Furthermore, histological analysis revealed no difference in the microglia-occupied brain areas or microglial morphologies between RAGE(-/-)/arcAbeta and arcAbeta mice. Conclusions: Together, our results indicate that while the absence of RAGE was associated with increased insulin-degrading enzyme activity in the brain, it was not sufficient to prevent or ameliorate cognitive deterioration, Abeta accumulation and microglial activation in the arcAbeta mouse model of AD.

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RAGE Does Not Affect Amyloid Pathology in Transgenic ArcAβ Mice

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Key Words
Alzheimer's disease · RAGE · Amyloid-β peptide · Insulin-degrading enzyme · Angiotensin-converting enzyme · Neprilysin · Microglia · Y maze

Abstract
Background: Alzheimer's disease (AD) is characterized by brain accumulation of the amyloid-β peptide (Aβ) that triggers a cascade of biochemical and cellular alterations resulting in the clinical phenotype of the disease. While numerous experiments addressed Aβ toxicity, the mechanisms are still not fully understood. The receptor for advanced glycation end products (RAGE) binds Aβ and was suggested to be involved in the pathological processes of AD. ***Objective:*** Our purpose was to assess the effect of *RAGE* deletion on Aβ-related pathology. ***Methods:*** We crossed RAGE knockout (RAGE$^{-/-}$) mice with transgenic mice harboring both the Swedish and Arctic Aβ precursor protein mutations (arcAβ mice). We assessed Aβ levels, Aβ brain deposition, Aβ-degrading enzyme activities, Aβ precursor protein expression and processing, number and morphology of microglia as well as cognitive performance of 6- and 12-month-old RAGE$^{-/-}$/arcAβ, RAGE$^{-/-}$, arcAβ and wild-type mice. ***Results:*** RAGE$^{-/-}$/arcAβ mice had significantly lower levels of SDS- and formic-acid-extracted Aβ in the cortex and hippocampus, with concomitantly increased activity of insulin-degrading enzyme at the age of 6 months. However, *RAGE* deletion could neither prevent the decline in cognitive performance nor the age-related cerebral accumulation of Aβ peptide. Furthermore, histological analysis revealed no difference in the microglia-occupied brain areas or microglial morphologies between RAGE$^{-/-}$/arcAβ and arcAβ mice. ***Conclusions:*** Together, our results indicate that while the absence of RAGE was associated with increased insulin-degrading enzyme activity in the brain, it was not sufficient to prevent or ameliorate cognitive deterioration, Aβ accumulation and microglial activation in the arcAβ mouse model of AD.

Introduction

Alzheimer's disease (AD), the most common form of dementia, is characterized by progressive cognitive deficits with underlying amyloid-β peptide (Aβ) accumulation, neuronal dysfunction and neuroinflammation. Both the evolution of neuronal impairment and inflammatory processes in AD pathology are suggested to be a result of receptor-mediated interactions with Aβ. One candidate receptor is the receptor for advanced glycation

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end products (RAGE), a multiligand receptor belonging to the immunoglobulin superfamily [1]. To determine the phenotype of brain β-amyloidosis in the absence of RAGE, we developed a mouse model, RAGE$^{-/-}$/arcAβ. ArcAβ mice, which overexpress the human β-amyloid precursor protein (APP) with both the Swedish and the Arctic mutations, show first cognitive deficits at the age of 6 months [2]. At that age, the animals have abundant Aβ intracellular punctate deposits in the cortex and hippocampus with no apparent Aβ plaque load. At 12 months of age, Aβ plaques and cerebral amyloid angiopathy (CAA) are prominent features. Therefore, we analyzed the RAGE$^{-/-}$/arcAβ mice at 6 and 12 months of age, and tested their cognitive abilities, biochemical and histopathological changes, including brain Aβ levels, plaque load, CAA and microglial morphology. RAGE$^{-/-}$/arcAβ animals showed significantly lower levels of SDS- and formic-acid-extractable Aβ in the cortex and hippocampus presumably related to concomitant increases in insulin-degrading enzyme (IDE) activity, as compared with arcAβ mice at the age of 6 months. However, these differences did not result in better cognitive performance and were no longer detectable 6 months later when massive Aβ accumulation occurs.

Materials and Methods

Animals

RAGE knockout (RAGE$^{-/-}$) mice [3] were crossed with arcAβ mice [2] to generate the RAGE$^{-/-}$/arcAβ mouse model. The arcAβ model is based on the overexpression of human APP 695 with the Swedish (K670N/M671L) and the Arctic (E693G) mutations in a single construct under the control of the prion protein promoter. There were 4 genotypes investigated in parallel: RAGE$^{-/-}$/arcAβ mice, their respective RAGE$^{-/-}$ littermates, arcAβ and their respective wild-type littermates. Six- and 12-month-old mice, 8–9 animals per genotype and age, were analyzed. Each animal group was balanced for gender. The mice were kept under standard housing conditions and a reversed 12-hour light/dark cycle, with free access to food and water. The animal experiments were approved by the Cantonal Veterinary Authority of Zurich. The abrogation of RAGE expression in RAGE$^{-/-}$ animals was demonstrated by Western blot of lung tissue, both directly by detecting no RAGE signal and indirectly by demonstrating reporter EGFR expression (online supplementary fig. 1, www.karger.com/doi/10.1159/000261723).

Y Maze

The spontaneous alternation rate was assessed using a Y-shaped plastic maze, with 40 × 20 × 10 cm arm sizes. During 5-min sessions, the sequences of arm entries were recorded; alternation was defined as successive entries into 3 arms, in overlapping triplet sets. The percent alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries – 2) × 100%. The behavioral testing was performed in the dark phase.

Tissue Preparation

Within 1 week of Y maze testing the mice were anesthetized with ketamine-xylazine cocktail (10 mg/kg, i.p.) and flush-perfused transcardially with ice-cold phosphate-buffered saline. Brains were rapidly removed and divided sagittally. One hemibrain was postfixed in phosphate-buffered 4% paraformaldehyde and paraffin-embedded; the other hemibrain was dissected into cortex, hippocampus and cerebellum snap-frozen and stored at –80°C for protein analysis.

Histology

Five-micrometer-thick sagittal sections were cut with a Leica RM 2135 microtome. The sections were pretreated with citrate buffer (20 min in microwave at 85 °C) followed by 95% formic acid (FA) [(5 min at room temperature (RT)], and blocked with 4% bovine serum albumin, 5% goat serum and 5% horse serum at RT for 1 h. The sections were subsequently incubated with primary antibodies overnight at 4°C at the following dilutions: mouse 6E10 (1:500); rabbit anti-Iba1 (1:500). Secondary fluorophore-conjugated antibodies were used for 2 h at RT. Thioflavin S staining was done according to standard protocol. Automated plaque and microglia counting was performed on 6 sections from each animal, spaced 50 μm apart, using the software Image J (http://rsb.info.nih.gov/ij/). The measurements were limited to the cortex, which was analyzed in the frontal and parietal regions, and the hippocampus.

Three-Step Aβ Extraction Protocol

Cortices were homogenized in 10 tissue volumes (w/v) of 1% Triton X-100 buffer (50 mM of Tris-HCl, pH 8.0/1% Triton X-100/complete protease inhibitor cocktail, Roche) with Teflon glass homogenizer (40 strokes) and subsequently centrifuged at 100,000 *g* for 1 h at 4°C. The supernatant was retained as the 1% Triton X-100 soluble fraction. The resulting pellet was homogenized in 10 tissue volumes (w/v) of 2% SDS buffer (50 mM of Tris-HCl, pH 8.0/2% SDS/2 mM EDTA/complete protease inhibitor cocktail EDTA-free, Roche) and centrifuged at 100,000 *g* for 1 h at 8°C. The ensuing supernatant was collected as the 2% SDS soluble fraction. Given the small amount of tissue obtained per hemibrain, mouse hippocampi were homogenized in 10 tissue volumes (w/v) of 1% Triton X-100, 2% SDS buffer (50 mM of Tris-HCl, pH 8.0/1% Triton X-100/2% SDS/complete protease inhibitor cocktail, Roche) with Teflon glass homogenizer (40 strokes) and subsequently centrifuged at 100,000 *g* for 1 h at 8°C. Two percent SDS pellets from cortices and hippocampi were further extracted with a minimum of 225 μl of 70% FA and spun at 100,000 *g* for 1 h at 8°C. The FA supernatant was collected by aspiration, avoiding the surface lipid layer and the acid insoluble bottom pellet. Aliquots of the 70% FA soluble fraction were lyophilized overnight in order to remove the acid and stored at –80°C.

Immunoblots

Tissue extracts were separated by SDS-PAGE using 10–20% Tricine gels (Invitrogen) and transferred onto nitrocellulose membranes. After an optional epitope retrieval and blocking in 5% milk TBS-T, the membranes were probed with 6E10 (1:500, Signet), 22C11 (1:1,000, Chemicon International), anti-APP C-terminal

(1:4,000, Sigma), anti-mouse/rat RAGE (1:500, R&D Systems; 1:1,000 rabbit polyclonal antibodies against RAGE V, C1 and C2 domains [4]) and anti-GFP (1:1,000, Roche) antibodies. Immunopositive bands were visualized by chemiluminescence (ECL, Amersham Biosciences) and subsequently quantified by densitometric analysis of their intensity levels. β-Tubulin was used as loading control (1:10,000, Sigma). The results were expressed as signal intensities normalized to values pertaining to arcAβ animals.

Aβ40 and Aβ42 ELISA of Cerebral Aβ

The Aβ40 and Aβ42 quantities in the above-mentioned fractions were determined using hAmyloid β40 and hAmyloid β42 ELISA kits (The Genetics Company AG, Switzerland).

Enzymatic Assays

Cortical tissue from 4 mice per genotype was pooled. The pooled tissue was homogenized in a 4-fold (w/v) volume of 20 mM Tris-HCl (pH 7.4), 0.5% Triton X-100, 10 μM PMSF. Homogenate was centrifuged at 13,000 rpm for 15 min at 4°C. Supernatant was used as a sample for the assay. The protein concentration was determined with the BCA Protein Assay (Pierce) and measured against bovine serum albumin standards.

IDE Activity Assay. The IDE activity in the supernatant was determined with the fluorometric Innozyme Insulysin/IDE Immunocapture Activity Assay Kit (Calbiochem, Merck) according to the manufacturer's instructions. The reaction time was 1 h. The IDE activity was expressed in nanograms/milliliter of crude cortical homogenates that were normalized to 2 mg/ml protein concentration.

Angiotensin-Converting Enzyme Activity Assay. The angiotensin-converting enzyme (ACE) activity in the supernatant against the synthetic substrate N-hippuryl-L-histidyl-L-leucine was determined using an ACE colorimetric kit (Buhlmann Laboratories AG, Schönenbuch, Switzerland). The reaction time was 3 h.

Neprilysin Activity Assay. The neprilysin enzyme activity was measured as described previously [5, 6], using a fluorometric assay for the generation of free dansyl-D-Ala-Gly (DAG) from *N*-dansyl-Ala-Gly-D-nitro-Phe-Gly (DAGNPG; Sigma), a fluorogenic substrate for neprilysin. Substrate solutions containing 1 mM of DAGPNG and 10 μM of enalapril (Sigma) in 20 mM of Tris HCl with and without the addition of 10 μM of NEP inhibitor phosphoramidon were prepared and preincubated at 37°C for 10 min. Enalapril, an ACE inhibitor, was added to prevent ACE-mediated DAGNPG cleavage. Fifty micorliters of the sample were incubated for 1 h with 100 μl of each substrate solution. The reaction was stopped by boiling for 10 min at 90°C. The samples were then spun for 5 min at 10,000 rpm. The fluorescence of the supernatants was measured at an emission wavelength of 562 nm and an excitation wavelength of 342 nm. The NEP activity was calculated as a difference in fluorescence between samples incubated with and without phosphoramidon, and expressed as relative fluorescence units.

Statistical Analysis

The data were analyzed using SPSS 14.0 (SPSS Inc., Chicago, Ill., USA). Differences between the means were assessed by Student's t test or 1-way ANOVA followed by Fisher's LSD or Tukey's post-hoc tests. Correlation studies were performed by parametric correlation and linear regression analysis. The null hypothesis was rejected at the 0.05 level.

Results

Absence of RAGE Reduced the Amount of Insoluble Aβ in 6-Month-Old ArcAβ Mice

In order to evaluate the influence of *RAGE* deletion (online suppl. fig. 1) on Aβ deposition, we examined the Aβ peptide levels in the brain regions relevant to cognition, i.e. the hippocampus and cortex. The Aβ levels were assessed with respect to the extraction conditions and the variant length (Aβ40 and Aβ42) using Western blot and C-terminus-specific ELISA. The brains were extracted sequentially in 1% Triton X-100, 2% SDS and 70% FA, to allow for the isolation of Aβ species that differ in the aggregation state and cellular/extracellular localization, with SDS-extractable Aβ pertaining to the membrane-enriched fraction and FA-extractable (insoluble) Aβ representing extracellular amyloid. Both the Western blot and ELISA approaches indicated significantly lower overall levels of accumulated Aβ in the cortex and hippocampus at the age of 6 months in RAGE$^{-/-}$/arcAβ animals compared to arcAβ mice with normal RAGE expression. Interestingly, the difference was due to the low Aβ levels extracted into SDS and FA, but not caused by Triton X-100-requiring peptide (tables 1 and 2). Despite the clear differences in the amount of extractable Aβ found by biochemical methods, histological analysis did not reveal any difference in Aβ-occupied brain areas at 6 months (fig. 1a), possibly due to the fact that at this age, visible plaque formation has not yet started in arcAβ mice [2]. At the age of 12 months, when Aβ deposition has progressed much more, biochemical and histopathological approaches revealed no more difference in the total Aβ load, nor Aβ40 and Aβ42 variants, in any of the fractions between RAGE$^{-/-}$/arcAβ and arcAβ mice (tables 1 and 2; fig. 1b). However, the Aβ40 level was significantly decreased in the serum of RAGE$^{-/-}$/arcAβ animals.

Given the possibility that the genetic manipulation might not only influence Aβ deposition and quantities but also morphological features of deposits, we examined both plaques and CAA but found no structural differences between RAGE$^{-/-}$/arcAβ and arcAβ mice (fig. 2 and 3). Plaques in RAGE$^{-/-}$/arcAβ animals, detected at 12 months (fig. 3a, g), had the same dense-core morphology as arcAβ mice (fig. 3b, d, h, j) and were also accompanied by severe CAA (fig. 3c, i).

RAGE Did Not Influence APP Expression and Processing

To determine whether the decreased amount of Aβ could be due to altered APP expression or processing

Table 1. Aβ levels measured by ELISA specific for Aβx-40 and Aβx-42

Age months	Genotype	Aβ	Cortex			Hippocampus		Serum pg/ml
			1% Triton ng/ml	2% SDS ng/ml	70% FA ng/ml	1% Triton, 2% SDS, ng/ml	70% FA ng/ml	
6	RAGE$^{-/-}$/arcAβ	Aβ40	4.52 ± 0.02	4.62 ± 0.09	1.59 ± 0.48*	3.47 ± 0.13	ND	ND
		Aβ42	6.37 ± 0.10	4.59 ± 0.13*	1.30 ± 0.50*	2.24 ± 0.04	ND	ND
	arcAβ	Aβ40	4.61 ± 0.03	6.02 ± 0.62	11.34 ± 3.53	3.56 ± 0.21	ND	ND
		Aβ42	6.68 ± 0.15	6.88 ± 0.87	6.52 ± 2.02	1.76 ± 0.33	ND	ND
12	RAGE$^{-/-}$/arcAβ	Aβ40	27.73 ± 8.93	143.48 ± 41.50	380.48 ± 111.04	20.38 ± 4.33	65.18 ± 18.37	258.51 ± 32.12*
		Aβ42	5.92 ± 1.07	50.14 ± 6.49	101.47 ± 42.07	5.56 ± 0.65	25.27 ± 5.35	ND
	arcAβ	Aβ40	15.71 ± 8.83	62.41 ± 12.58	402.39 ± 140.74	11.80 ± 2.88	96.35 ± 33.93	432.70 ± 59.93
		Aβ42	4.01 ± 1.19	44.38 ± 5.90	97.64 ± 33.33	5.29 ± 0.92	36.13 ± 10.80	ND

Values are means ± SE. ND = Not detectable. * $p < 0.05$.

Table 2. Monomeric Aβ levels determined by densitometric analysis of Western blot assay with 6E10 antibody

Age months	Genotype	Cortex			Hippocampus	
		1% Triton	2% SDS	70% FA	1% Triton, 2% SDS	70% FA
6	RAGE$^{-/-}$/arcAβ	96.0 ± 32.5	10.3 ± 3.7**	7.7 ± 3.9***	67.0 ± 23.2	3.36 ± 1.8*
	arcAβ	100.0 ± 6.5	100.0 ± 35.0	100.0 ± 23.1	100.0 ± 30.1	100.0 ± 55.3
12	RAGE$^{-/-}$/arcAβ	222.1 ± 88.8	164.7 ± 48.6	101.6 ± 24.7	76.2 ± 18.9	83.6 ± 25.7
	arcAβ	100.0 ± 33.0	100.0 ± 12.4	100.0 ± 18.3	100.0 ± 15.7	100.0 ± 20.5

Data are mean relative intensity ± SE. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

caused by the absence of RAGE, we examined the levels of full-length APP (APP), soluble APP (sAPP) as well as C-terminal fragments produced by β-secretase (CTF-β) by Western blot using 6E10, 22C11 and anti-APP C-terminal antibodies. There was no difference in APP, sAPP and CTF-β levels according to the densitometric analysis of immunoblots upon normalization to β-tubulin in the cortex and the hippocampus between the 2 transgenic groups (fig. 4).

RAGE$^{-/-}$/ArcAβ Mice Showed Increased Aβ-Degrading Enzymatic Activity

Our finding that the cerebral levels of SDS- and FA-soluble Aβ were reduced in the brains of 6-month-old mice without any change in APP processing suggested that the activity of 1 or more Aβ-degrading proteases might be increased. To test this hypothesis, we examined the enzymatic activity of 3 proteases in the cortices of 6- and 12-month-old animals. Analysis of IDE activity revealed significant main effects of the APP transgene [$F(1,16) = 28.218$, $p = 0.001$] and of the RAGE knockout condition [$F(1,16) = 37.102$, $p < 0.001$], with no interaction, indicating their additive effect. At the age of 6 months, IDE in RAGE$^{-/-}$/arcAβ had a significantly higher activity than in arcAβ mice ($p = 0.05$), RAGE$^{-/-}$ mice ($p = 0.11$) and wild-type animals ($p < 0.001$; fig. 5a). However, at the age of 12 months the difference between RAGE$^{-/-}$/arcAβ and arcAβ was not statistically significant; RAGE$^{-/-}$/arcAβ animals had an increased activity of the enzyme only in comparison with the wild-type animals ($p = 0.043$). There was no significant difference in the activity of 2 other Aβ-degrading enzymes, ACE and neprilysin, among the 4 groups (fig. 5b, c).

Absence of RAGE Did Not Ameliorate Cognitive Impairment

To clarify whether the reduced amount of Aβ in RAGE$^{-/-}$/arcAβ mice at 6 months would result in less se-

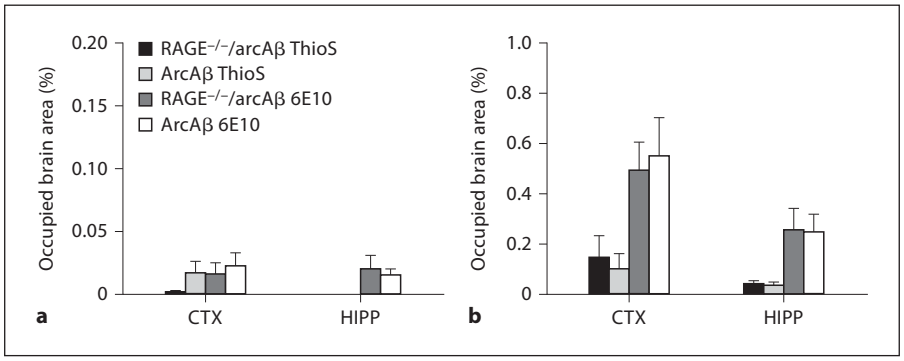


Fig. 1. Percentage of brain areas occupied by Aβ deposits, detected by thioflavin S (ThioS) and 6E10 staining, at the age of 6 (**a**) and 12 (**b**) months. There was no statistically significant difference between the 2 APP transgene-bearing groups. **a** No thioflavin-S-positive deposits were detected in the hippocampus at the age of 6 months. Values are means ± SE, n = 8–9 mice per group. CTX = Cortex; HIPP = hippocampus.

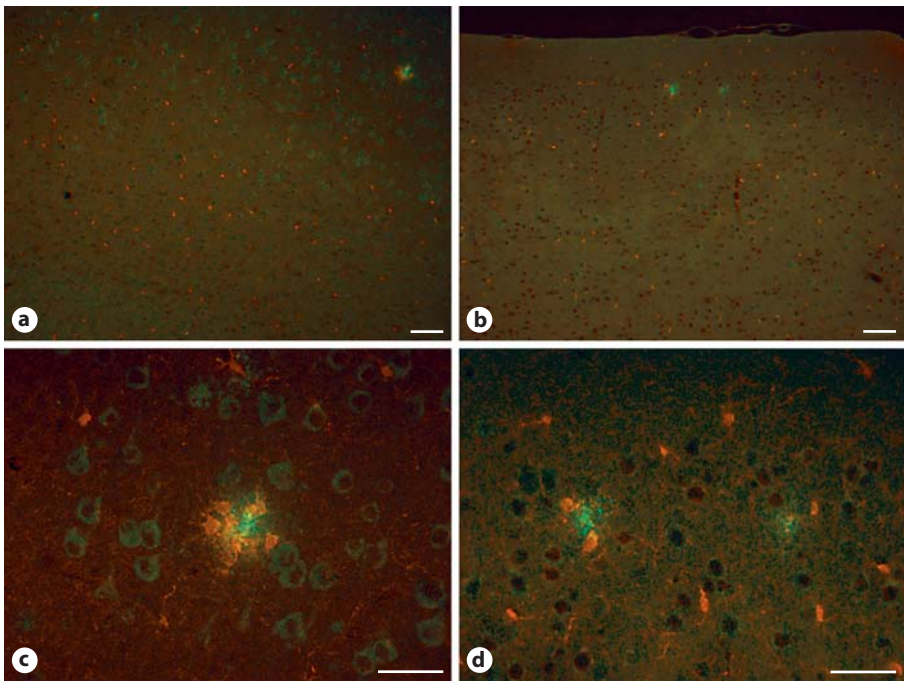


Fig. 2. The lack of RAGE does not change Aβ deposition at the age of 6 months. In the overview of cortical sections of 6-month-old RAGE$^{-/-}$/arcAβ (**a**) and arcAβ (**b**) mice there were no plaques or CAA detected, just a few 6E10-stained (green) Aβ deposits that started to attract microglia (Iba1; red), as visible in (**c**) and (**d**) with enlarged details from the overview images. Scale bar: 100 μm (**a**, **b**); 50 μm (**c**, **d**).

vere cognitive deficits that had previously been described in arcAβ mice, the different genotype groups were tested in the Y maze, a working memory test. There was no significant difference in Y maze performance between the RAGE$^{-/-}$/arcAβ and arcAβ animal groups at the age of 6 and 12 months. Although they had less SDS- and FA-soluble Aβ load at 6 months, the RAGE$^{-/-}$/arcAβ mice had the same extent of cognitive deficits as the arcAβ animals. This impairment was not due to the lack of RAGE, since the RAGE$^{-/-}$ mice performed equally well as the wild-type animals (fig. 6).

Cognitive Impairment Correlated with Triton-X-Extractable Aβ

Given the cognitive deficits in the animals harboring the APP transgene, we evaluated whether the extent of accumulated Aβ was related to the degree of cognitive performance in individual mice. There was a significant negative correlation between the percent alternation in the Y maze and monomeric Aβ cortical levels present in 1% Triton X-100 fraction determined by densitometric analysis of Western blot with 6E10 antibody but not with the Aβ levels in other fractions or in the hippocampus. Additionally, there was no significant correlation between Y-maze performance and the levels of 2 length variants, Aβ40 or Aβ42. The association between soluble Aβ load in the cortical area and working memory impairment was significant at the age of 6 months for both RAGE$^{-/-}$/arcAβ ($p = 0.0146$) and arcAβ mice ($p = 0.043$) (fig. 7a), and the r values obtained for the 2 groups were not significantly different. At 12 months of age, the negative correlation was only significant for the RAGE$^{-/-}$/arcAβ mice ($p = 0.020$; fig. 7b).

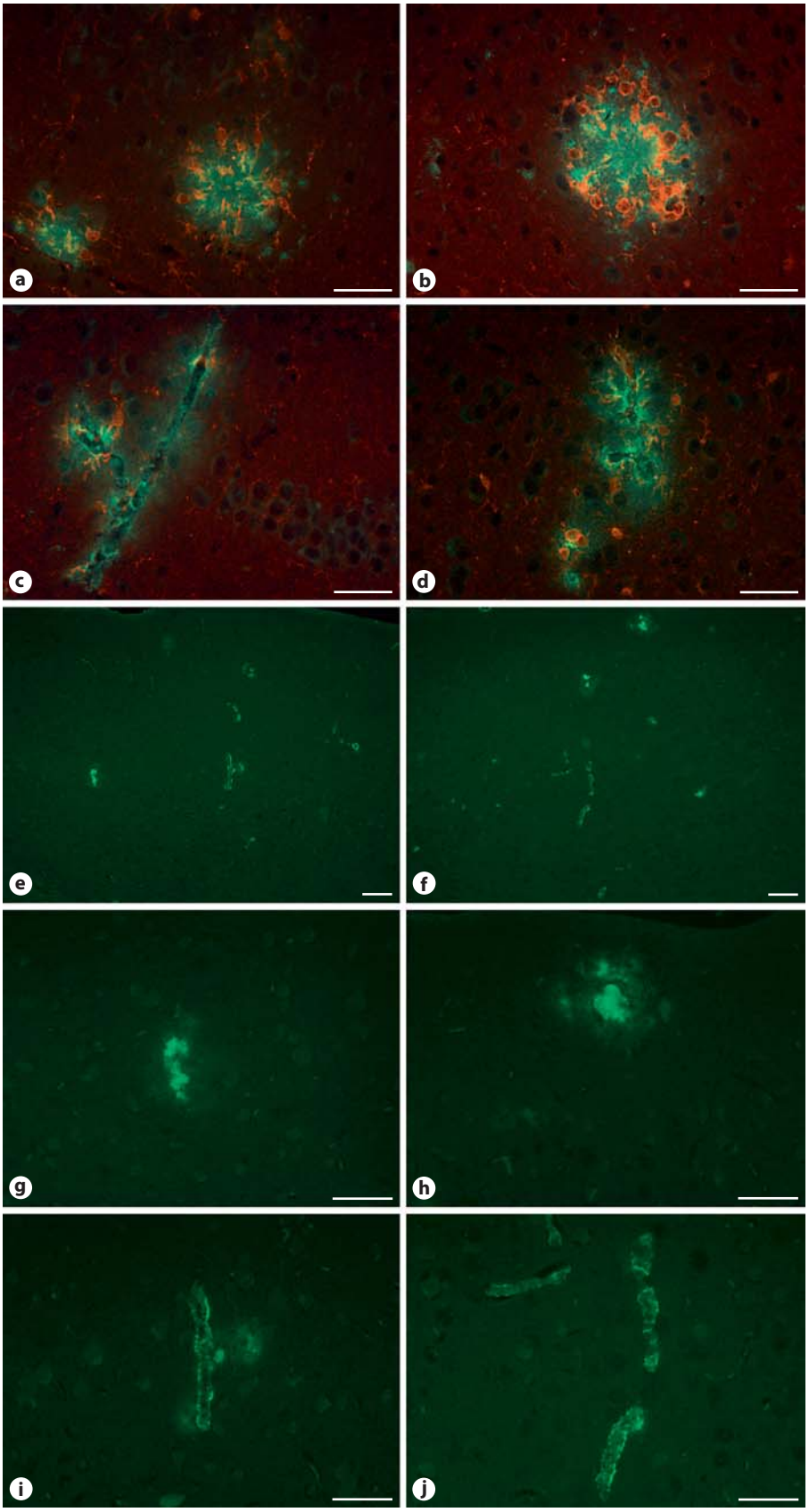


Fig. 3. Plaque dense-core morphology and CAA at 12 months in the cortex and hippocampus of RAGE$^{-/-}$/arcAβ (**a**, **c**, **e**, **g**, **i**) and arcAβ (**b**, **d**, **f**, **h**, **j**). **a–d** 6E10 staining (green), paralleled with the Iba1 staining of microglia [31], revealing plaque morphology (**a**, **b**) and CAA (**c**, **d**). **e–j** Thioflavin-S-stained overview images of mouse cortices (**e**, **f**) and their respective enlarged details demonstrating dense-core plaques (**g**, **h**) and CAA (**i**, **j**). Scale bar: 100 μm (**e**, **f**); 50 μm (**a–d**, **g–j**).

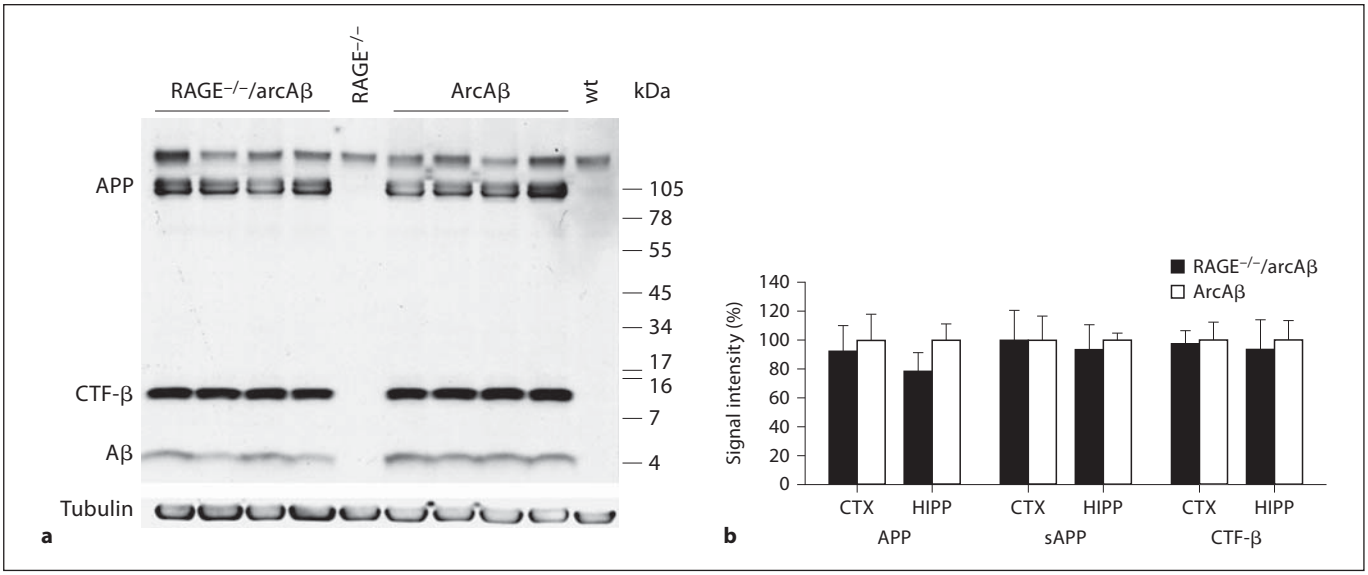


Fig. 4. Western-blot-based characterization of APP, sAPPα and CTFs in 6- and 12-month-old RAGE$^{-/-}$/arcAβ mice. **a** Representative 6E10 immunoblot from 1% Triton X-100 cerebral cortex homogenates from 6-month-old animals. **b** Densitometric analysis of APP, sAPP and CTF-β signal intensities in 6E10, 22C11 and APP C-terminal immunoblots, respectively, normalized to β-tubulin. Each value represents the mean ± SE of 8–9 six-month-old mice for each brain region analyzed. CTX = Cortex; HIPP = hippocampus; wt = wild type.

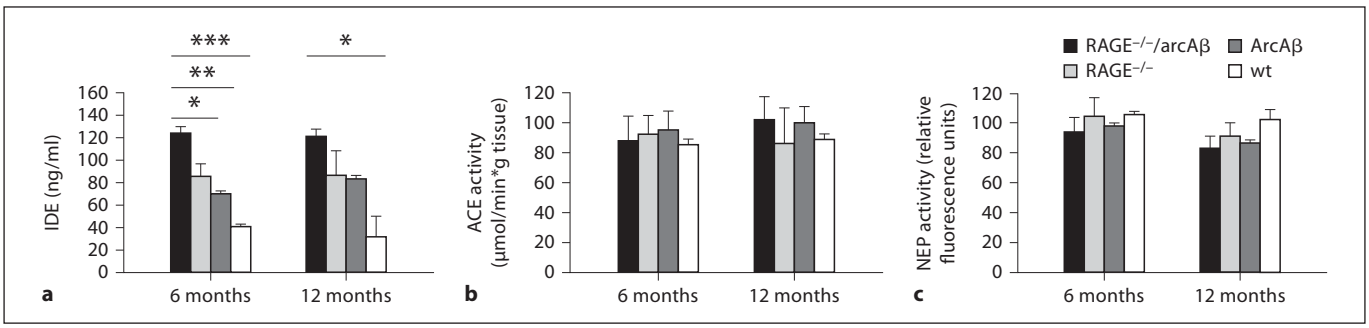
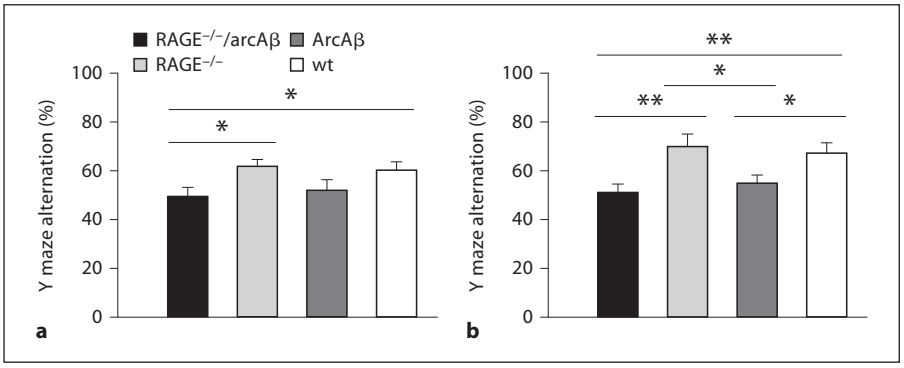


Fig. 5. Enzyme activity measurements in crude cortical homogenates. **a** IDE activity was significantly increased in the RAGE$^{-/-}$/arcAβ cortices at the age of 6 months in comparison with the 3 other genotypes. **b** There was no difference in ACE activity among the animal groups. **c** No difference was detected in neprilysin activity among the different genotypes. Values are means ± SE, n = 8–9 mice per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: 1-way ANOVA followed by Tukey's post-hoc analysis. wt = Wild type.

Fig. 6. Behavioral performance in the Y maze, a working memory test measuring spontaneous alternation behavior, at the age of 6 (**a**) and 12 (**b**) months. RAGE$^{-/-}$/arcAβ had impaired working memory in comparison to control animals already at the age of 6 months. Values are means ± SE, n = 8–9 mice per group. * $p < 0.05$; ** $p < 0.01$: 1-way ANOVA followed by Fisher's LSD post-hoc analysis. wt = Wild type.



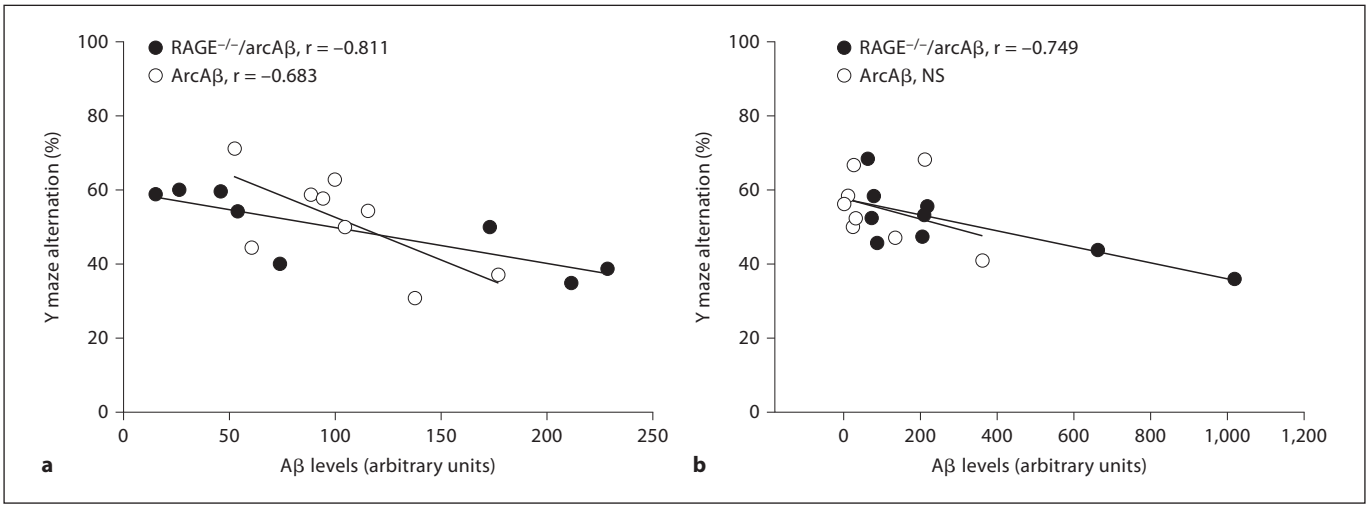


Fig. 7. Inverse correlation between Y maze performance and Aβ cortical levels present in 1% Triton X-100 fraction determined by densitometric analysis of 6E10 immunoblot. **a** At 6 months, the levels of soluble monomeric Aβ in the cortical fraction were significantly negatively correlated with the percent alternation in the Y maze in both RAGE$^{-/-}$/arcAβ ($p = 0.0146$) and arcAβ mice ($p = 0.043$). **b** At 12 months of age the negative correlation was significant only for the RAGE$^{-/-}$/arcAβ mice ($p = 0.020$).

Lack of RAGE Did Not Alter the Amount of Microglia

No difference in the microglia-occupied brain area or microglial morphology was observed in RAGE$^{-/-}$mice in comparison to wild-type animals at both examined ages (fig. 8c, d). Also, these animals did not differ from RAGE$^{-/-}$/arcAβ and arcAβ mice with respect to the microglia-occupied brain area at the age of 6 months, when no dense-core plaques or CAA can be demonstrated yet (fig. 8a). At 12 months, there was a significant equal increase in Iba1-occupied brain area in both RAGE$^{-/-}$/arcAβ and arcAβ mice when compared to APP non-transgenic animals (fig. 8b), due to the formation of plaques and CAA that attract microglia (fig. 4a–d).

Discussion

Several lines of evidence suggested a role of the RAGE receptor in AD pathogenesis with different underlying processes proposed. Studies in mice and cell culture indicated that RAGE promotes Aβ accumulation through 2 different mechanisms. First, RAGE is thought to mediate Aβ peptide influx into the central nervous system through the blood-brain barrier [7, 8], thus increasing the amount of Aβ in the brain. The second mechanism leading to enhanced Aβ accumulation via RAGE proposes a direct stimulation of BACE1 expression, the secretase involved in Aβ production [9]. In addition, RAGE is assumed to promote AD pathology by activating microglia, with subsequent induction of chemotaxis, cell proliferation, as well as ROS and cytokine production [1, 10, 11]. To better understand the in vivo role of RAGE in AD pathology, we generated and characterized arcAβ mice lacking RAGE. These animals had an increased IDE activity during the first 6 months of life and were protected from Aβ accumulation only to a certain extent. There were lower levels of Aβ extractable by SDS and FA (insoluble Aβ) in the brains of RAGE$^{-/-}$/arcAβ mice at the age of 6 months, but this effect disappeared by the age of 12 months, most probably due to the constant peptide production overwhelming the clearance process. In addition, the observed IDE upregulation resulted from an additive effect of *RAGE* deletion and the APP transgene or probably Aβ accumulation, as previously suggested [12, 13]. Although it presumably decreased initial Aβ build-up, the IDE-mediated clearance process did not prevent cognitive decline and eventually became inefficient at the age of 12 months given the similar amounts of cerebral Aβ in both genotypes. ArcAβ mice have been previously shown to generate Aβ only in the nervous system [2], thus precluding the relevance of external Aβ influx into the brain to the development of brain pathology in this mouse model. In addition, our study showed significantly lower serum levels of Aβ40 in RAGE$^{-/-}$/arcAβ mice than in the arcAβ animals at the age of 12 months, contradicting the possible relevance of Aβ transport across the blood-brain

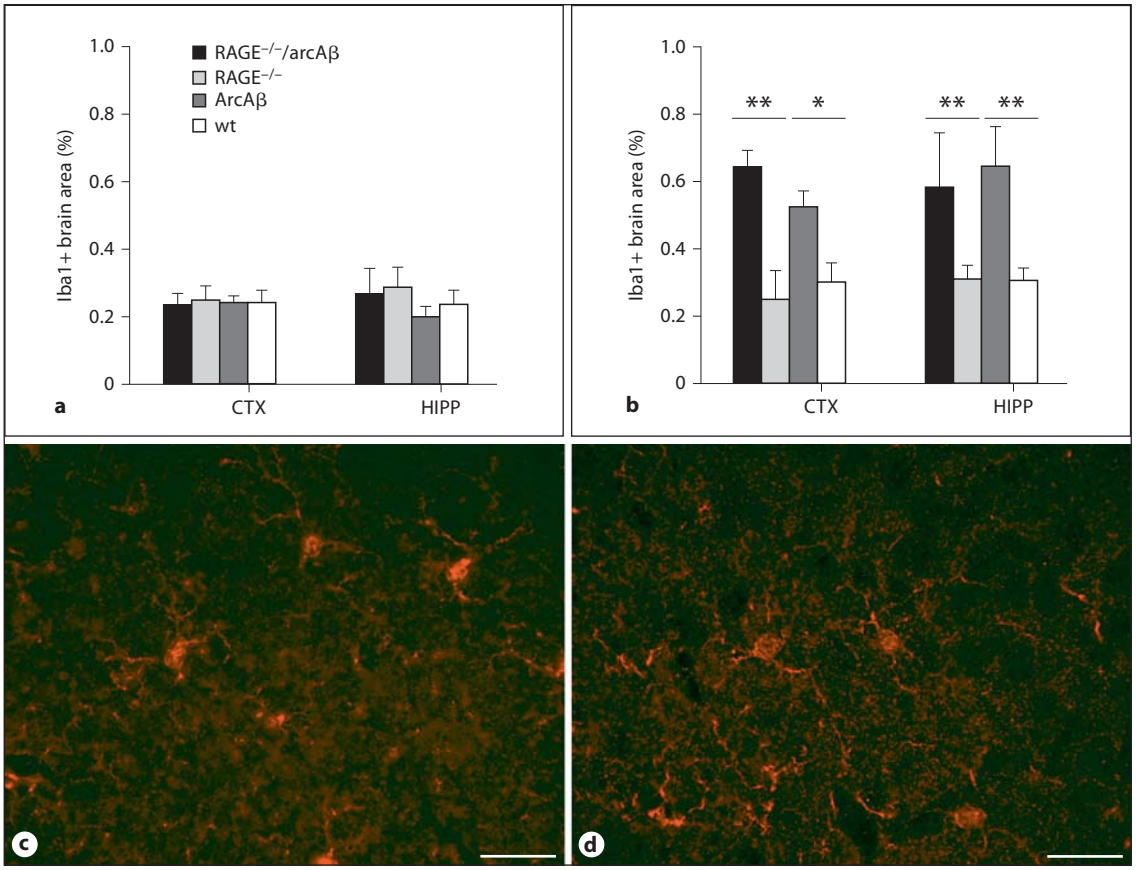


Fig. 8. Percentage of brain areas occupied by microglia (Iba1+ cells) at the age of 6 (**a**) and 12 (**b**) months. The significant increase in both RAGE$^{-/-}$/arcAβ mice and arcAβ observed at 12 months of age was associated with the plaque accumulation. Values are means ± SE, n = 8–9 mice per group. There was no difference in the microglia-occupied brain area or microglial morphology between RAGE$^{-/-}$ (12 months; **c**) and wt (12 months; **d**) animals. Scale bar: 30 μm. CTX = Cortex; HIPP = hippocampus; wt = wild type. * $p < 0.01$, ** $p < 0.001$: 1-way ANOVA followed by Tukey's post-hoc analysis.

barrier for cerebral Aβ accumulation. RAGE involvement in APP processing via BACE did not seem to be plausible in the arcAβ mice either given the identical levels of CTF-β observed in both genotypes. Similarly, our study did not reveal any change in microglia number or morphology in animals lacking RAGE. The RAGE$^{-/-}$/arcAβ mice had comparable brain areas occupied by microglia in both age groups. Additionally, there was a marked activation of microglia evident in parallel with the onset of fibrillar plaques in both genotypes, which would not favor an in vivo role of RAGE to promote microglia activation in AD either. This is in agreement with the previous findings in APP23 mice, whereby the majority of activated microglia, present in the vicinity of amyloid plaques, did not upregulate RAGE expression [14].

Differences observed between previous studies and the results presented here may be explained by confounding factors not accounted for in previously applied experimental settings, methodologies and animal models. These include APP transgenic mice overexpressing RAGE or bearing dominant-negative RAGE (DN-RAGE), DN-RAGE and wild-type mice injected or infused with Aβ, as well as APP transgenic or Aβ-infused mice which were treated with truncated, soluble form of RAGE, sRAGE [7, 15, 16]. Remarkably, sRAGE-treated animals show greater protection compared to RAGE-null (RAGE$^{-/-}$) animals against deleterious effects of inflammation [17], diabetic nephropathy and concomitant VEGF expression [18], or diabetic neuropathy [19], conditions in which RAGE is suggested to be a key pathology-mediating molecule. In addition, exogenous sRAGE blocks inflammation even in RAGE$^{-/-}$ animals [17], which altogether suggests involvement of sRAGE in additional pathways linked to inflammatory response. Endogenous sRAGE is

produced by alternative splicing of the RAGE mRNA as well as by proteolytic cleavage [20, 21]. According to the studies conducted with exogenous sRAGE, endogenous sRAGE was supposed to play an antagonistic role by competing with cell surface RAGE, thus inhibiting RAGE-induced signaling [7, 17, 22]. The physiologic concentrations of circulating sRAGE, found in diabetic patients, are much lower than required to scavenge accumulating ligands [23]. It is, however, possible that RAGE and DN-RAGE overexpression [15] leads to increased levels of sRAGE with protective properties. Additionally, mechanisms accounting for the absence of pathological effects in APP mice expressing DN-RAGE could be similar to the more overt protection observed in DN-RAGE mice compared to $RAGE^{-/-}$ animals in the context of atherosclerosis [24].

No reduction in Aβ levels was observed in Triton X-100 brain extract, the only Aβ fraction correlating with Y maze performance. Such a correlation is in line with the previous AD research pointing to the causative role of Aβ oligomers present in the soluble brain extracts [25, 26]. At the age of 12 months, when the Aβ load increased in all fractions in both genotypes, such a correlation was not detected in arcAβ mice, possibly because of different susceptibility to soluble Aβ in this group of animals. The same extent of cognitive impairment observed in $RAGE^{-/-}$/arcAβ mice as in arcAβ mice is also challenging the hypothesis about RAGE contribution to brain Aβ-related pathophysiology. This is consistent with the recent study investigating a relationship between the psychomotor or cognitive performance and amounts of RAGE increasing with age in several brain regions which failed to detect any association between the variables [27]. Moreover, in the same study there was no correlation between the age-related accumulation of advanced glycation end products, which are major RAGE ligands, and RAGE expression in the mouse brain. Another report about enhanced Aβ deposition in the mouse brain did not find any parallel significant change in RAGE expression [28].

The observed significant upregulation of IDE in $RAGE^{-/-}$/arcAβ mice at the age of 6 months suggested that IDE expression and RAGE signaling function are associated. The underlying network linking the 2 molecules has not been unraveled. Recently, it has been described that while the 'anti-inflammatory' cytokines IL-4 and IL-13 increase IDE expression [29], 'pro-inflammatory' IFN-γ and TNF-α have an opposite effect [30]. Deletion of *RAGE* and removal of its proinflammatory influence may thus push the balance towards the anti-inflammatory pathways and promote IDE expression. In conclusion, a general contribution of RAGE to microglial activation, Aβ intracerebral accumulation or cognitive impairment in the arcAβ mice seems to be unlikely. The initial increase in IDE activity, however, along with the reduced brain Aβ levels during the initial 6 months of life may permit an unexpected role of RAGE in regulating Aβ proteolysis.

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